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## Recherches préliminaires sur la structure de la partie glucidique des caséino-glycopeptides\*

### Résumé

Quelques expériences préliminaires concernant le groupement prothétique glucidique des caséino-glycopeptides sont décrites: l'acide sialique et le galactose occupent une position «externe» par rapport à la galactosamine sans doute liée à la partie peptidique.

La caséine entière de vache privée de sa fraction  $\alpha$  est pratiquement dépourvue de sucres<sup>1</sup>. Ce fait suggère que probablement tous les sucres se trouvent localisés dans la caséine  $\alpha$  qui est la seule fraction glycoprotéique importante de la caséine. Sous l'effet de la présure, à pH 6,8, même en absence d'ions Ca, la caséine  $\alpha$  donne naissance à deux fractions: l'une, insoluble, appelée paracaséine  $\alpha$ ; l'autre, très soluble, appelée «caséino-glycopeptide  $\alpha$ ». Rappelons que la présure agit sur la seule fraction  $\alpha$  de la caséine entière, qui est ainsi le véritable substrat de l'enzyme<sup>3</sup>. Le caséino-glycopeptide  $\alpha$  contient la majeure partie des sucres de la caséine, à savoir, du galactose, de la galactosamine et de l'acide neuraminique; le caséino-glycopeptide est donc la fraction glucidique la plus importante de la caséine<sup>4</sup>. L'étude de son groupement prothétique glucidique peut être abordée en travaillant soit avec la caséine soit avec le caséino-glycopeptide: quelques expériences préliminaires ont été effectuées en faisant appel à des méthodes chimiques (action de H<sub>2</sub>SO<sub>4</sub> 0,1 N; action de l'acide périodique) ou à une méthode enzymatique (action de la neuraminidase).

### Composition en sucres des CGP

Le tableau 1 indique la composition en sucres des caséino-glycopeptides (CGP) de vache (provenant de la

caséine  $\alpha$ ), de brebis (provenant de la caséine entière) et de chèvre (provenant de la caséine entière): les rapports moléculaires ont été calculés en admettant que les poids moléculaires des CGP sont de l'ordre de 8000. Les CGP de brebis et de chèvre contiennent un mélange des acides N-acétyl- et N-glycolyl-neuraminiques.

Tableau 1. Composition en sucres de trois caséino-glycopeptides en % de substance sèche;  $r$  = rapport moléculaire calculé en fixant le poids moléculaire des CGP égal à 8000

Caséino-glycopeptide	Galactosamine*	Galactose**	Acide sialique***
	%	$r$	%
CGP $\alpha$ de vache	6,5	2,9	6,0 2,7 10,2 2,8
CGP de brebis	2,4	1,0	2,1 0,9 1,0 0,3
CGP de chèvre	3,4	1,5	3,6 1,6 2,8 0,7

\* Selon RONDELLE et MORGAN<sup>5</sup>.

\*\* Selon SCHULTZE et coll.<sup>6</sup>

\*\*\* Exprimé en acide N-acétylneuraminique (PM 309), dosage selon WARREN après hydrolyse acide<sup>7</sup>.

### Action de la neuraminidase sur la caséine $\alpha$ et les CGP

Lorsque l'on fait agir la neuraminidase de *V. cholerae*, à pH 6 (solution tampon de phosphates 0,03 M) pendant 3 heures à 37°, sur une solution à 1% de caséine  $\alpha$  de vache ou de l'un des trois CGP mentionnés dans le tableau 1, la totalité des acides neuraminiques est libérée: l'acide neuraminique occupe donc une position terminale.

### Action du périodate de K sur la caséine $\alpha$

A une solution à 1% de caséine  $\alpha$  de vache dans une solution tampon de phosphates 0,05 M; pH 7; contenant

\* Reçu le 10 octobre 1964.

<sup>1</sup> C. ALAIS, Thèse Sci., Paris 1962; C. ALAIS et P. JOLLÈS, *Lait* 44 (1964) 138.

<sup>2</sup> P. JOLLÈS, C. ALAIS et J. JOLLÈS, *Biochim. Biophys. Acta* 51 (1961) 309.

<sup>3</sup> D. F. WAUGH et P. H. VON HIPPEL, *J. Amer. Chem. Soc.* 78 (1956) 4576.

<sup>4</sup> C. ALAIS et P. JOLLÈS, *Biochim. Biophys. Acta* 51 (1961) 315.

<sup>5</sup> C. J. RONDELLE et W. T. J. MORGAN, *Biochem. J.* 61 (1955) 586.

<sup>6</sup> H. E. SCHULTZE, R. SCHMIEDBERGER et R. HAUPP, *Biochem. Z.* 329 (1958) 490.

<sup>7</sup> L. WARREN, *J. Biol. Chem.* 234 (1959) 1971.

du KCl (0,15 M), on ajoute du périodate de K (35 mg  $\text{KIO}_4$  pour 100 mg de caséine  $\alpha$ ); 80% de l'acide sialique sont détruits en 30 minutes à température ordinaire; par contre, les teneurs en galactose et en galactosamine demeurent constantes.

#### Action de la neuraminidase suivie de celle du périodate de K sur la caséine $\alpha$

On fait agir cette fois le périodate de K sur une solution de caséine  $\alpha$  préalablement incubée avec de la neuraminidase, donc privée de l'acide sialique terminal. Après 30 minutes à température ordinaire, 40% de galactose ont disparu: par contre, la teneur en galactosamine demeure constante. Il semble donc que ce soit le galactose qui après le départ de l'acide sialique occupe une position «externe».

#### Action de l'acide sulfurique 0,1 N sur les CGP

Les CGP de vache ( $\alpha$ ), de brebis et de chèvre sont hydrolysés pendant 6 heures à 100° avec  $\text{H}_2\text{SO}_4$  0,1 N<sup>8,9</sup>. L'hydrolysat est dessalifié puis chromatographié sur papier Whatman n° 1 dans le solvant *n*-butanol-acide acétique-eau (4 : 1 : 5, v/v). Deux substances ont pu être identifiées avec le réactif à l'oxalate d'aniline: le galactose libre (très abondant) et une substance peu abondante de  $R_{\text{GlcNH}_2} = 0,82$ . Il n'a pas été possible de caractériser de la galactosamine libre.

D'autre part, la substance de  $R_f = 0$  a été élue du papier puis analysée. Elle contient de nombreux acides aminés et de la galactosamine (liée aux acides aminés); par contre, il n'a pas été possible de déceler la présence de galactose. Ces expériences montrent que c'est le galactose qui, après la destruction de l'acide sialique, occupe une position «externe»; par contre, c'est la

<sup>8</sup> J. MONTREUIL et A. CHOSSON, *C. R. Acad. Sci.* 255 (1962) 3071.

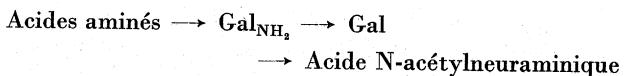
<sup>9</sup> J. MONTREUIL, G. SPIK et A. CHOSSON, *C. R. Acad. Sci.* 255 (1962) 3493.

galactosamine qui semble être directement liée à la partie peptidique.

#### Conclusion

La labilité particulière des acides sialiques et du galactose permet de placer ces composés en position «externe» par rapport à la galactosamine plus «interne». Ces trois glucides se trouvent en proportions équimoléculaires dans le CGP de vache. Dans les deux autres CGP, la galactosamine et le galactose se trouvent en proportions équimoléculaires, par contre, les résultats du dosage des acides sialiques indiquent qu'il y a 2 à 3 fois moins de résidus de ces composants; mais il s'agit de substances particulièrement labiles: le dosage, dans des matières qui en contiennent très peu, manque de précision.

Des recherches ultérieures devront déterminer si le groupement prosthétique du CGP de vache est formé par trois enchaînements identiques:



ou par une seule chaîne contenant les 9 résidus glucidiques groupés 3 par 3. La nature de la liaison entre la partie peptidique et la galactosamine qui, d'après nos expériences, ne semble pas être du type ester, est également en cours d'étude.

En ce qui concerne les CGP de brebis et de chèvre, de nouvelles déterminations des composants glucidiques seront faites afin de préciser les rapports moléculaires.

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## Studies on the Transfer of Some Flavour Compounds to Milk

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To determine what flavour compounds could be transferred to milk via the digestive route, series of aliphatic alcohols, aldehydes, ketones and esters were fed into the rumen of a cow. The milk samples were analyzed by means of a gas chromatograph. Alcohols with an odd number of carbon atoms, lower ketones and esters (except those methyl and ethyl esters of fatty acids with an odd number of carbon atoms tested) were found to enter the milk to such a degree that they gave distinct flavour defects to milk. Aldehydes, with one exception, and some unsaturated alcohols and ketones were transferred to milk only in traces or not at all.

Flavour threshold values of some aliphatic alcohols, ketones and esters in milk are given.

It is a well known fact that certain fodder plants and silages can give rise to characteristic flavour defects in milk. For example an onion-like off-flavour was noticed in milk after feeding cows with some *Allium*-species, *Melilotus albus* gave an aroma of coumarin and *Trifolium pratense* a clover-like taste. Likewise some fodder plants of the family *Cruciferae* gave typical off-flavours to milk. Sometimes a grass-like off-flavour appears when the cows are moved to pasture feeding in the springtime. Silages can also impart an extremely bad off-flavour to milk if repulsive flavour substances have been formed in the silage by the effect of micro-organisms, as happens if the pH is not sufficiently low.

The milk flavours can be classified into two groups: those flavour compounds which are transferred from fodder to milk via the organism and those which are formed in the organism (in the rumen and/or in the metabolic processes in the liver or mammary gland) from carbohydrates, amino or fatty acids and other chemical compounds in the fodder.

Recently Dougherty and co-workers<sup>1,2</sup> have performed some experiments with cows having ruminal and tracheal fistulas to determine what chemical compounds could be transmitted to milk through the lungs or the rumen or both. They found that if sufficient amounts (usually such enormous amounts as 25–50 ml) of the substances tested (esters, alcohols, ketones, aldehydes,

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*Table 3. Transfer of some aliphatic compounds to milk via the rumen.*

Substance	Amount fed, g	Concentr. at the maximum, $\mu\text{g}/1 \text{milk}$	Total amount transferred to milk, $\mu\text{g}$	Percentage of total amount fed
<i>Alcohols:</i>				
<i>n</i> -Pantan-1-ol	2	380	250	0.013
<i>n</i> -Hexan-1-ol	2	30	20	0.001
<i>n</i> -Heptan-1-ol	2	120	100	0.005
<i>n</i> -Octan-1-ol	2	?	—	—
<i>n</i> -Nonan-1-ol	2	75	100	0.005
<i>cis</i> Hex-3-en-1-ol	2	45	25	0.0013
<i>trans</i> Hex-3-en-1-ol	3	25	15	0.0005
<i>dl</i> Oct-1-en-3-ol	1	20	15	0.0015
<i>Aldehydes:</i>				
2-Methyl-propanal	2	1500	600	0.03
Hexanal	2	15	10	0.0005
Heptanal	2	20	10	0.0005
Octanal	2	30	10	0.0005
<i>Ketones:</i>				
Pantan-2-one	2	1300	1000	0.05
Hexan-2-one	2	1100	800	0.04
Heptan-2-one	2	750	250	0.013
Octan-2-one	2	490	150	0.0075
Nonan-2-one	2	150	50	0.0025
Decan-2-one	2	60	30	0.0015
Undecan-2-one	2	40	25	0.0013
Octan-3-one	2	50	25	0.0013
Oct-1-en-3-one	2	—	—	—
<i>Esters:</i>				
Methyl esters of $\text{C}_6 - \text{C}_{10}$ fatty acids	à 2	—	—	—
Ethyl esters of $\text{C}_5, \text{C}_7, \text{C}_9$ fatty acids	à 2	—	—	—
Ethyl butanoate	2	120	120	0.006
Ethyl hexanoate	2	60	60	0.003
Ethyl octanoate	2	120	100	0.005
Ethyl decanoate	2	120	100	0.005
Butyl 2-methyl-propanoate	1	300	160	0.016
Butyl butanoate	1	160	120	0.012
Butyl 3-methyl-butanoate	1	140	90	0.009
Butyl pentanoate	1	200	150	0.015
Butyl hexanoate	1	260	170	0.017
Butyl octanoate	1	110	75	0.0075

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In a previous paper<sup>3</sup> from this laboratory a semiquantitative gas chromatographic micromethod was described for the determination of some flavour compounds in liquid foodstuffs. By this method it is possible to demonstrate the presence of as little as 5 µg of added flavour compound in one liter of milk (0.005 ppm), which concentration is considerably lower than the flavour threshold concentration of most aliphatic compounds in milk (Table 1). Since the percentage recovery from milk which contains fat decreases very rapidly with the increase of the molecular weight of the flavour compounds we have performed several distillations with known quantities of substances added to obtain the relevant percentage recovery for various types of organic compounds. The average values are presented in Table 2. As can be seen, the

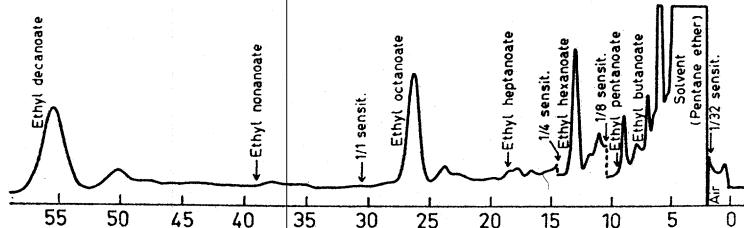
*Table 2.* Percentage recovery of flavour compounds added to milk (4–5 % fat).

Total number of carbon atoms	Percentage of aldehydes, ketones and esters	Percentage of alcohols
4	5–15	35–45
5	35–45	65–75
6	55–65	75–85
7	70–80	65–75
8	75–85	45–55
10	55–65	—
12	15–25	—

maximum recovery (70–85 %) is obtained with aldehydes, ketones and esters having 7 or 8 carbon atoms and by alcohols having 5–7 carbon atoms. The low boiling compounds (b.p. < 80°C) are lost almost completely in the concentration procedure with pentane-ether extraction, and the higher-boiling compounds are held in the fat.

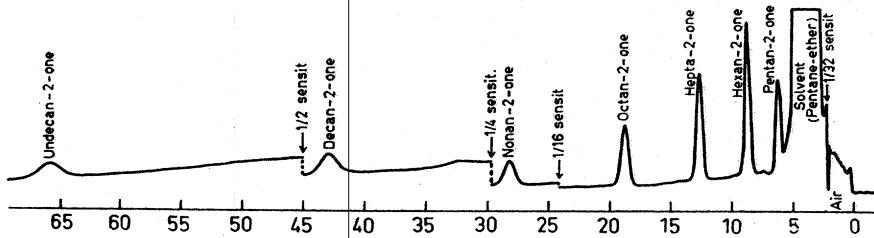
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*Fig. 3.* Gas chromatogram of the milk obtained 2 h after feeding of ethyl esters of *normal* fatty acids ( $C_4-C_{10}$ ), 2 g of each. The same column and conditions as in Fig. 2.

the milk to a considerably greater degree than the others. Of the ethyl esters only those with an even number of carbon atoms in the fatty acids enter the milk. An alternation like this is not observed with methylketones (Fig. 4) or



*Fig. 4.* Gas chromatogram of the milk obtained 2 h after feeding of *n*-alkan-2-ones ( $C_6-C_{11}$ ), 2 g of each. Silicone grease column, 6 m, i.d. 4.5 mm, 175°C, 45 ml/min of  $N_2$ .

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The percentage transfer varies within wide limits. The methyl esters of  $C_6-C_{10}$  fatty acids are not transferred to milk in detectable amounts and only traces of aldehydes, except 2-methylpropanal, were found in the milk. The ketones, alcohols (odd C-numbers) and esters are transferred to such a degree that they can give distinct flavour defects to milk, although the percentage transfer is as low as 0.01–0.05 %. Lillard *et al.*<sup>4</sup> have observed an additive effect if several flavour compounds are present at the same time, so that also lower concentrations than the threshold value can give flavour defects to milk. Nawar and Fagerson<sup>5</sup> pointed out the potential significance of sub-threshold amounts of some methyl ketones and concluded that a synergistic effect was obtained by combining the compounds. In the experiments of Guadagni *et al.*<sup>6</sup> the effect of mixtures containing substances of a single class or different classes was additive. For instance, a mixture of 10 saturated aldehydes containing each compound at only about 10 % of its threshold concentration gave an odour.

The unsaturated alcohol, oct-1-en-3-ol, a compound isolated from some clover species,<sup>12</sup> also was found to enter the milk. The maximum concentration,

Most of the substances tested were transferred to the milk, the maximum concentration being reached in general after 2 h. The higher molecular weight compounds tend, however, to reach this point somewhat later (after 4 h). The concentration then rapidly decreases so that after 8–10 h only about 10 % of the maximum concentration is present.

A typical feeding test with a mixture of *cis* and *trans* hex-3-en-1-ol is seen in Fig. 1. As can be seen, the amount of the *cis* form, the so called "leaf alcohol",

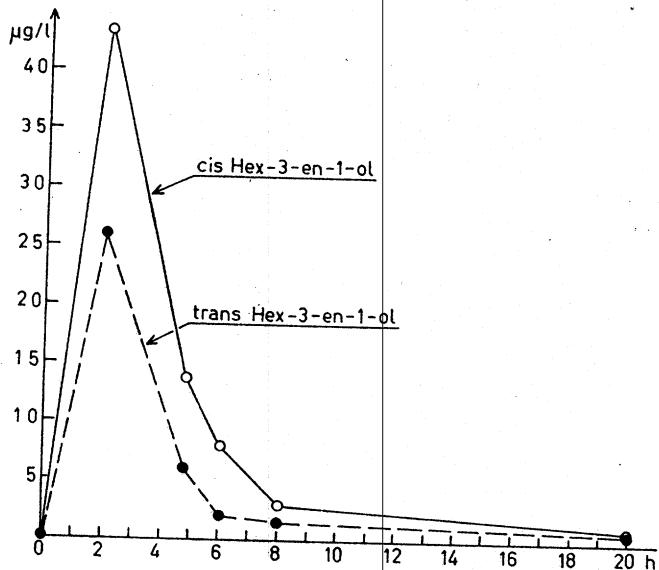


Fig. 1. Feeding test with a mixture (5 g) of *cis* (40 %) and *trans* (60 %) hex-3-en-1-ol. Ord.: transfer of both components to the milk,  $\mu\text{g/l}$  milk; absc.: time after feeding. 1. *cis* Hex-3-en-1-ol. 2. *trans* Hex-3-en-1-ol.

transferred to the milk is more than double that of the *trans* isomer (Table 3). Similar interesting features can also be observed in the feeding tests with normal aliphatic alcohols (Fig. 2) and ethyl esters of aliphatic fatty acids (Fig. 3). The alcohols with an odd number of carbon atoms are transferred to

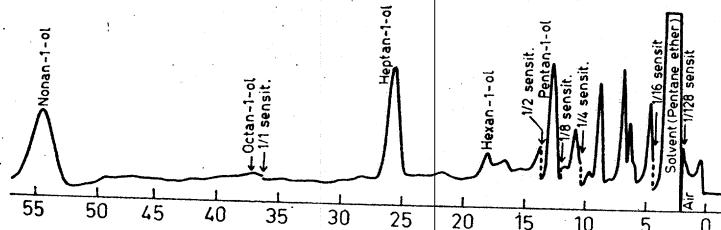


Fig. 2. Gas chromatogram of the milk obtained 2 h after feeding of *n*-alkan-1-ols ( $\text{C}_5$ – $\text{C}_9$ ), 2 g of each. Polyethyleneglycol column, 6 m, i.d. 4.5 mm,  $175^\circ\text{C}$ , 45 ml/min of  $\text{N}_2$ .

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20 µg/l, was obtained after 2–4 h. In organoleptic tests, however, no detectable off-flavour was observed. According to Stark and Forss<sup>7</sup> a possible precursor for the so called "metallic off-flavour" (identified as oct-1-en-3-one) is oct-1-en-3-ol, which was also isolated from dairy products ("mushroom compound"). Accordingly, it is possible that oct-1-en-3-ol is transferred from fodder to milk and the metallic off-flavour formed in milk and especially in dried milk might be formed by oxidation of this compound to the corresponding ketone. After feeding the cows with aftermath, a peak with the identical retention time as the synthetical oct-1-en-3-ol appears in the gas chromatogram of the milk. The corresponding ketone, oct-1-en-3-one, which was also isolated from the flowers of *Trifolium repens* and *Tr. hybridum*<sup>13</sup> did not enter the milk via the digestive route. Some unidentified lower molecular weight compounds appear, however, after the feeding of oct-1-en-3-one in the gas chromatogram of the milk. This ketone was found to be sensitive to acids and it disappears obviously very quickly in the rumen.

EXPERIMENTAL

The flavour threshold values were determined according to Patton and Josephson.<sup>8</sup>

The vacuum steam distillation and solvent extraction method for the isolation and concentration of the flavour compounds for gas chromatographic analyses is described in an earlier paper.<sup>3</sup> A "Fraktometer 116 E" of the Perkin Elmer Co. with FID and a sample inlet valve<sup>3</sup> were used in the experiments.

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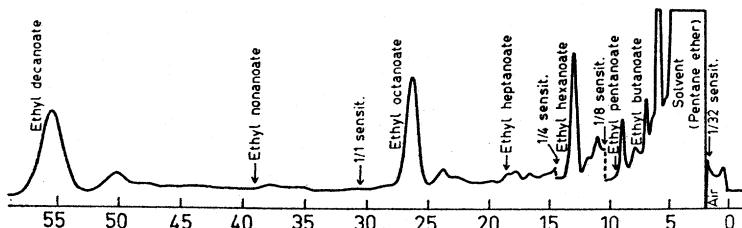


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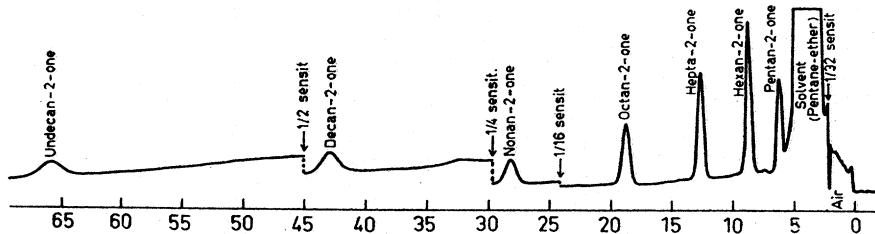


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The unsaturated alcohol, oct-1-en-3-ol, a compound isolated from some clover species,<sup>12</sup> also was found to enter the milk. The maximum concentration,

Most of the substances tested were transferred to the milk, the maximum concentration being reached in general after 2 h. The higher molecular weight compounds tend, however, to reach this point somewhat later (after 4 h). The concentration then rapidly decreases so that after 8–10 h only about 10 % of the maximum concentration is present.

A typical feeding test with a mixture of *cis* and *trans* hex-3-en-1-ol is seen in Fig. 1. As can be seen, the amount of the *cis* form, the so called "leaf alcohol",

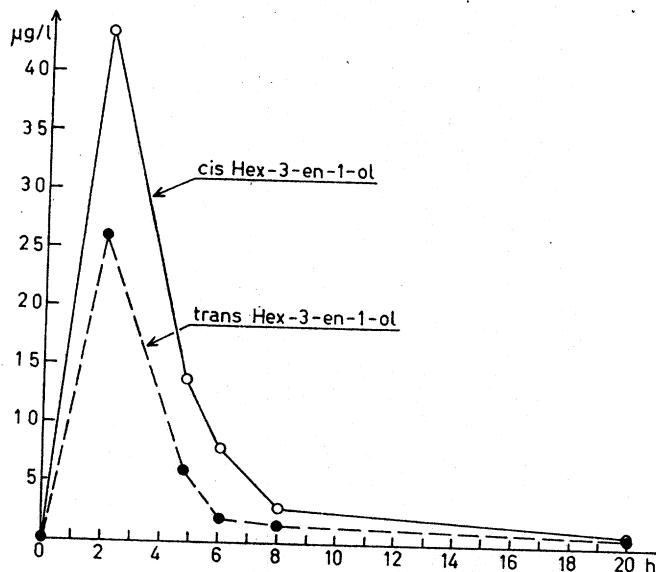


Fig. 1. Feeding test with a mixture (5 g) of *cis* (40 %) and *trans* (60 %) hex-3-en-1-ol.  
Ord.: transfer of both components to the milk, µg/l milk; absc.: time after feeding. 1. *cis* Hex-3-en-1-ol. 2. *trans* Hex-3-en-1-ol.

transferred to the milk is more than double that of the *trans* isomer (Table 3). Similar interesting features can also be observed in the feeding tests with normal aliphatic alcohols (Fig. 2) and ethyl esters of aliphatic fatty acids (Fig. 3). The alcohols with an odd number of carbon atoms are transferred to

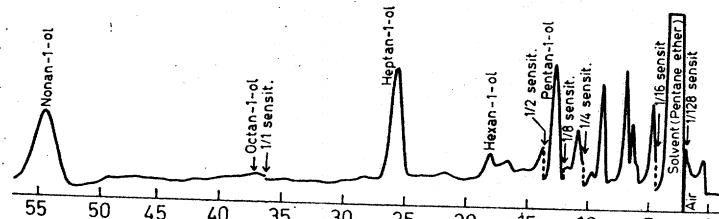


Fig. 2. Gas chromatogram of the milk obtained 2 h after feeding of *n*-alkan-1-ols (C<sub>5</sub>–C<sub>9</sub>), 2 g of each. Polyethyleneglycol column, 6 m, i.d. 4.5 mm, 175°C, 45 ml/min of N<sub>2</sub>.

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20 µg/l, was obtained after 2—4 h. In organoleptic tests, however, no detectable off-flavour was observed. According to Stark and Forss<sup>7</sup> a possible precursor for the so called "metallic off-flavour" (identified as oct-1-en-3-one) is oct-1-en-3-ol, which was also isolated from dairy products ("mushroom compound"). Accordingly, it is possible that oct-1-en-3-ol is transferred from fodder to milk and the metallic off-flavour formed in milk and especially in dried milk might be formed by oxidation of this compound to the corresponding ketone. After feeding the cows with aftermath, a peak with the identical retention time as the synthetical oct-1-en-3-ol appears in the gas chromatogram of the milk. The corresponding ketone, oct-1-en-3-one, which was also isolated from the flowers of *Trifolium repens* and *Tr. hybridum*<sup>13</sup> did not enter the milk via the digestive route. Some unidentified lower molecular weight compounds appear, however, after the feeding of oct-1-en-3-one in the gas chromatogram of the milk. This ketone was found to be sensitive to acids and it disappears obviously very quickly in the rumen.

EXPERIMENTAL

The flavour threshold values were determined according to Patton and Josephson.<sup>8</sup>

The vacuum steam distillation and solvent extraction method for the isolation and concentration of the flavour compounds for gas chromatographic analyses is described in an earlier paper.<sup>3</sup> A "Fraktometer 116 E" of the Perkin Elmer Co. with FID and a sample inlet valve<sup>3</sup> were used in the experiments.

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